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(57) Abstract

Modifying TNF with polyethyleneglycol (PEG) having an approximate weight average molecular weight in the range of about 10,000 to about 40,000, preferably in the range of about 20,000 to 30,000, significantly increases the circulating half-life of the TNF. As a result, lower doses of the TNF may be administered to effectively treat tumors, with fewer, accompanying adverse side effects to the patient.

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MODIFIED TUMOR NECROSIS FACTOR

Related Applications

This application claims the benefit of U.S. Provisional Patent Application Serial No. 60/035,521, filed on January 15, 1997.

Field of the Invention

This invention is directed, *inter alia*, to tumor necrosis factor modified with polyethylene glycol having a molecular weight in the range of 10,000 to 40,000 and methods for treating tumors using such modified tumor necrosis factor.

Background of the Invention

Malignant melanoma (stage 3) is a fatal disease killing most patients within one year of diagnosis. The incidence of melanoma is rapidly increasing in the United States and is even higher in other countries, such as Australia. Effective treatments for patients suffering from melanoma are urgently needed.

Kidney cancer currently kills approximately 13,000 individuals in the United States each year. This form of cancer is frequently not detected until it is well advanced. The only form of treatment that significantly affects a patient's prognosis is surgical resection of the affected organ. Unfortunately, because this type of cancer is highly metastatic, complete removal of all the metastasis is difficult, if not impossible.

Colon cancer is one of the most prevalent forms of cancer and currently kills approximately 140,000 individuals in the United States each year. Although there have been a large number of traditional chemotherapeutic drugs developed to treat this disease, long term survival (defined as the percentage of patients surviving five years or more) has not appreciably changed in the last four decades. Furthermore, all of the

traditional chemotherapeutic drugs developed are highly toxic, have deleterious and often fatal side effects, and are expensive. A curative, non-toxic treatment for this disease is urgently needed.

A hallmark of melanomas, kidney and colon tumors is that these tumors quickly develop resistance to traditional chemotherapies. Even though patients may initially respond to chemotherapeutic treatment, drug-resistant tumors quickly develop and ultimately kill the patient. An alternative way to treat these tumors would be to identify an "Achilles Heel" in the tumors and to develop therapies that would selectively treat that target. One such potential target has been identified. Specifically, it has been noted that all three of these types of tumors require extensive vascularization of each of the metastacies in order for the cancers to grow. Therefore, one would predict that a therapeutic agent which would inhibit the vascularization of these tumors may provide a unique means of treating these tumors.

Tumor necrosis factor (TNF) is one of many cytokines, which are a group of diverse proteins produced by leukocytes and related cells, having immunostimulating activity. TNF was originally named for its ability to cause tumors to necrosis. There are at least two different mechanisms by which TNF is believed to kill tumors. The first is by a direct effect on the tumor itself. Alternatively, TNF can selectively disrupt the vascularization of tumors. In an early manuscript describing TNF, Carswell and Old reported that the METH A tumor cells were completely resistant to TNF *in vitro*. *J. Proc. Natl. Acad. Sci USA*, 72:3666-3670 (1975). However, METH A tumors in mice were extremely sensitive to TNF *in vivo*. This was believed to be because TNF selectively disrupted the vascularization of these tumors. It was later shown that some as-yet-unidentified factor is released by some tumors that renders the normal vascular endothelial cells adjacent to them susceptible to TNF killing. In short, TNF kills these tumors not by directly killing the tumor cells, but rather by killing the normal vascular endothelial cells that line the blood vessels that provide the tumor with blood, oxygen and other nutrients necessary to live and grow.

Unfortunately, early clinical trials attempted to develop TNF as a direct tumoricidal agent. When used in this fashion, large doses of TNF needed to be injected as it quickly (less than 20 minutes) leaves the circulation. Furthermore, these high doses of TNF induced "shock"-like symptoms characterized by a precipitous drop in blood

pressure. An alternative way of using TNF would be to formulate it so that it remains in the circulation. By doing this, the TNF would have more time to interact with the vasculature of the tumor and would have sufficient time to disrupt the blood supply to the tumor.

Several other therapeutic proteins have been formulated with polyethylene glycol (PEG) so that they would circulate longer and remain in the vasculature. These proteins include asparaginase, adenosine deaminase, and super oxide dismutase. See, for example, Harras, J.M., in "Polyethylene Glycol Chemistry: Biotechnical and Biochemical Applications," Plenum Press (1992). A group of Japanese investigators have previously described that TNF could be formulated with certain PEG and that the resulting material had substantially increased circulating half-life and greater anti-tumor activity. Tsutsumi, Y., et al., *Jap. J. Cancer Res.*, **85**:9-12 (1994); Tstutsumi, Y., et al., *Jap. J. Cancer Res.*, 85:1185-1188 (1994); Tsutsumi, Y., et al., *Jap. J. Cancer Res.*, 87:1078-1085 (1997). These investigators used only PEG with a molecular weight of 5000 coupled to primary amines on TNF with a succinimidyl succinate linker.

Summary of the Invention

It has now been found, surprisingly, that modifying TNF with polyethylenegycol (PEG) having an approximate weight average molecular weight in the range of about 10,000 to about 40,000, preferably in the range of about 20,000 to 30,000, significantly increases the circulating half-life of the TNF and also enhances the tumoricidal activity of the TNF. For example, the serum half life of TNF has been increased, by such modification, from as little as 20 minutes up to fifteen days, and the anti-tumor ED50 has been decreased from as much as 1000-3000 IU to as little as 10-50 IU. As a result of the modification, lower doses of the TNF may be administered to effectively treat tumors, with fewer, accompanying adverse side effects to the patient.

This invention, therefore, relates to modified TNF, wherein said modification comprises covalently bonding to said TNF, either directly or through a biocompatible linking agent, one or more PEG molecules having an approximate weight average molecular weight in the range of about 10,000 to about 40,000. Preferably, the TNF is modified with five to twelve of the PEG molecules, more preferably, with about five to nine PEG molecules.

This invention also relates to a method of treating a patient suffering from a tumor by administering to said patient a therapeutically effective amount of said modified TNF.

This invention further relates to a method of enhancing the circulating half life of TNF comprising modifying said TNF by covalently bonding to it between about five and twelve PEG molecules having an approximate weight average molecular weight in the range of about 10,000 to about 40,000.

This invention further relates to a method of enhancing the tumoricidal activity of TNF comprising modifying said TNF by covalently bonding to it between about five and twelve PEG molecules having an approximate weight average molecular weight in the range of about 10,000 to about 40,000.

Description of the Drawings

Figure 1 is a graph depicting the circulating half life in mouse serum of native TNF-α (open circles), SS 5,000 MW PEG-TNF-α (closed circles), and 20,000 MW PEG-TNF-α (open triangles).

Figure 2 is a graph depicting the circulating half life in mouse serum of native TNF-α (open circles), SS 5,000 MW PEG-TNF-α (closed circles), SS 12,000 MW PEG-TNF-α (closed triangles), SS-20,000 MW PEG-TNF-α (open triangles), NHS 12,000 MW PEG-TNF-α (closed squares), and NHS 20,000 MW PEG-TNF-α (open squares).

Detailed Description of the Invention

"Tumor necrosis factor" or "TNF" as used herein encompasses either naturally derived protein, such as isolated human or mouse TNF proteins, or protein produced using recombinant technology, such as recombinant murine TNF and recombinant human TNF. Although the TNF-α protein is preferred, the term "TNF" also encompasses TNF-β protein. The terms also encompass TNF proteins that have been mutated by deletion or alteration of amino acids without significantly impairing biological activity (e.g., as non-limiting examples, delete amino acids 212-220 or change lysine at 248, 592, 508 to alanine).

"Polyethylene glycol" or "PEG" refers to mixtures of condensation polymers of ethylene oxide and water, in a branched or straight chain, represented by the general formula $H(OCH_2CH_2)_nOH$. "Polyethylene glycol" or "PEG" is used in combination with a numeric suffix to indicate the approximate weight average molecular

weight thereof. For example, PEG 5,000 refers to polyethylene glycol having an approximate weight average molecular weight of about 5,000; PEG 12,000 refers to polyethylene glycol having an approximate weight average molecular weight of about 12,000; and PEG 20,000 refers to polyethylene glycol having an approximate weight average molecular weight of about 20,000. Such polyethylene glycols are available from several commercial sources, and are routinely referred to, as indicated above, by their weight average molecular weights.

"Melanoma" may be a malignant or benign tumor arising from the melanocytic system of the skin and other organs, including the oral cavity, esophagus, anal canal, vagina, leptomeningers, and/or the conjunctivae or eye. The term "melanoma" includes, for example, acral-lentginous melanoma, amelanotic melanoma, benign juvenile melanome; lentigo maligna melanoma, malignant melanoma, nodular melanoma, subungual melanoma and superficial spreading melanoma.

"Patient" refers to an animal, preferably a mammal, more preferably a human.

"Biocompatible" refers to materials or compounds which are generally not injurious to biological functions and which will not result in any degree of unacceptable toxicity, including allergenic and disease states.

"Circulating half life" refers to the period of time, after injection of the modified TNF into a patient, until a quantity of the TNF has been cleared to levels one half of the original peak serum level. Circulating half life may be determined in any relevant species, including humans or mice.

"Covalently bound" as used herein refers to a covalent bond linking the TNF protein to the PEG molecule, either directly or through a linker.

According to this invention, TNF is modified with polyethylene glycol having an approximate weight average molecular weight in the range of 10,000 to 40,000, preferably in the range of 20,000 to 30,000. Generally, polyethylene glycol with a molecular weight of 30,000 or more is difficult to dissolve, and yields of the formulated product are greatly reduced. The polyethylene glycol may be branched or straight chain, but is preferably a straight chain.

The polyethylene glycols may be bonded to the TNF through biocompatible linking groups. As discussed above, "biocompatible" indicates that the compound or

group is non-toxic and may be utilized *in vitro* or *in vivo* without causing injury, sickness, disease or death. PEG may be bonded to the linking group, for example, via an ether bond, an ester bond, a thiol bond, or an amide bond. Suitable biocompatible linking groups include, for example, an ester group, an amide group, an imide group, a carbamate group, a carboxyl group, a hydroxyl group, a carbohydrate, a maleimide group (including, for example, succinimidyl succinate (SS), succinimidyl propionate (SPA), succinimidyl carboxymethylate (SCM), succinimidyl succinamide (SSA), or N-hydroxy succinimide (NHS), an epoxide group, an oxycarbonylimidazole group (including, for example, nitrophenyl carbonate (NPC) or trichlorophenyl carbonate (TPC)), a trysylate group, an aldehyde group, an isocyante group, a vinylsulfone group, a tyrosine group, a cysteine group, a histidine group or a primary amine. Preferably, the biocompatible linking group is an ester group and/or a maleimide group and bonds to the TNF through a primary amine on the TNF protein. More preferably, the linking group is SS, SPA, SCM, SSA or NHS; with SS being the most preferred.

Alternatively, TNF may be coupled directly to PEG (i.e., without a linking group) through an amino group, a sulfhydryl group, a hydroxyl group, or a carboxyl group.

Methods for covalently bonding TNF to PEG, directly or via a biocompatible linking group, are known in the art, as described, for example, in Harras, J.M., in "Polyethylene Glycol Chemistry: Biotechnical and Biochemical Applications," Plenum Press (1992), the disclosure of which is herein incorporated by reference. It is preferred that the TNF protein be covalently bonded to five to twelve PEG molecules. Methods for determining the number of PEG molecules bonded to the protein are known in the art, for example, Habeeb, A.F.S.A., *Anal. Biochem.*, **14**:328-339 (1966); Harras, J.M., *supra.*, herein incorporated by reference. The number of PEG molecules bonded to TNF will vary according to the linking group utilized, the length of reaction, and the molar ratios of TNF and PEG utilized in the reaction.

As one skilled in the art would recognize, the modified TNF of this invention may be administered in a number of ways, for example, orally, intranasally, intraperitoneally, parenterally, intravenously, intralymphatically, intratumorly, intramuscularly, interstitally, intrarterially, subcutaneously, intraocularly, intrasynoially, transepithelially, and transdermally. A therapeutically effective amount of one of the

modified compounds of the present invention is an amount effective to inhibit tumor growth, and that amount may vary according to the method of administration. Generally, effective doses should be in the range of about 0.001 to 0.1 mg/kg, once a week. The modified TNF may be formulated with pharmaceutically acceptable carriers and diluents, as known in the art. For example, for intravenous? administration, the modified TNF may be mixed with a phosphate buffered saline solution, or any other appropriate solution known to those skilled in the art, prior to injection. Tests have shown that the modified TNF is particularly effective in treating melanoma, colon cancer, kidney cancer and breast cancer tumors.

The invention is further demonstrated in the following examples, which are for purposes of illustration, and are not intended to limit the scope of the present invention.

TNF used in the experiments described below was of mouse and human origin. The human TNF (whole protein) was produced in *E. coli,* and murine TNF as well as the human TNF mutants were produced in *Pichea pastoris*. Recombinant TNF was produced in *E. coli* or *Pichea* using methods similar to those described in Pennica, D., et al., *Nature*, **312**:724-729 (1981); Streekishna, K., et al., *Biochemistry*, **28**:4117-4125 (1989). The mouse TNF was produced in *E. coli* and in *Pichea*.

Example 1

Specific Activity of TNF-α

Prior to pegylation, mature tissue necrosis factor (TNF-α) (human recombinant) was tested using a L929 cytotoxicity assay as described below. The specific activity of the TNF-α was 10^6 I.U. units per milligram. Densitometry of an SDS-PAGE gel showed that the material was 99% pure.

The material (1 ml at 16.8 mg/ml, determined by the method of Bradford, M.M., *Anal. Biochem.*, **72**:248-254 (1976), with a bovine serum albumin (BSA) standard, Peterson modification) was concentrated to approximately 0.1 ml using a 3,000 kDa cutoff Centricon. This material was then diluted to 4 ml with 100 mM phosphate buffer, pH 8.0 and reconcentrated to 0.1 ml. This procedure was repeated twice, and the resulting material was finally collected in a total volume of 1 ml of the same buffer.

The protein concentration was then determined by the method of Bradford, above. BSA was used as a standard, and about 0.6 mg of protein was recovered.

Pegylation

PEGylating reactions were performed using the general methods described in Harras, J.M., cited above. To TNF (1mg/ml in 100 mm phosphate buffer, pH 7.2-7.5), the SS-PEG or NHS-PEG? was added at a 10 to 50 molar excess and mixed for one hour at room temperature. The specific pH and molar ratio used varied with the reactivity of the PEG and had to be empirically determined. The PEG-TNF was separated from unreacted PEG and TNF by ultra filtration using a 100 kDa cut off filter. In each of the modifications referenced in this example, the TNF was modified with five to nine molecules of PEG.

Purity of the PEG-TNF was assessed by SDS-PAGE and the percent of primary amines modified by this procedure was determined using florescamine as described by S.J. Stocks (Anal. Biochem. **154**:232 (1986)). SDS-PAGE results indicated that very little, if any, native TNF-α remained in the preparation after pegylation.

In vitro activity of the PEG-TNF-α

The PEG-TNF-α were examined for in vitro cytotoxic activity using the L-929 cytotoxicity assay performed according to the procedure set forth below, with the exception that the passage number of the cells was not known. The specific activity of the TNF-α starting material was 1.5 x 10^6 units/mg or about one half of the original specific activity measurement. The differences in specific activity were attributed to the use of a different protein determination method and the unknown passage number of the cells.

The specific activity of the SS 5,000 MW PEG-TNF-α was 0.7 x 10^6 units/mg, or about one half the specific activity of the native material. The specific activity of the SS-20,000 MW PEG-TNF-α was 0.8 x 10^6 units/mg, a value similar to the SS 5,000 MW PEG-TNF-α.

Lethality of the PEG-TNF-α

As a screen, two C57 bl6 mice (female, 20-25 g) were injected intraperitoneally (i.p.) with either native TNF-α or SS-PEG-TNF-α and survival of the animals was monitored. The doses used were 1, 5, and 10 thousand units of activity.

With native TNF-α, the following results were obtained:

10,000 I.U. - both mice dead the next morning

5,000 I.U. - one mouse dead next morning; the second mouse in obvious distress (hair ruffled and little movement) and dead after 2 days

1,000 I.U. - one mouse dead the next morning; the second mouse in distress (hair ruffled and little movement) and in such poor condition after 2 days that it was euthanized

With the SS-PEG-TNF-α, all mice at all doses remained in good health for two weeks following injection. Behavior was normal, as was eating and drinking. There was no change in coat (fur was not ruffled). All of the mice were euthanized 15 days post-injection.

Determination of serum half life of PEG-TNF-α

An ELISA assay for human TNF obtained from Genzyme was used. The kit was used as suggested by the manufacturer. Mice were injected with either TNF-α or PEG-α (100 units) i.p., and approximately 25 μl of serum was collected from retro-orbital bleeds at the times indicated in Fig. 1. A total of 5 mice (female, C57 bl6 mice, 20-25g) were in each group.

The native TNF-α (open circles) was cleared very fast, and the only data point above baseline was 30 minutes post-injection.

The SS 5,000 MW peg-TNF-α (closed circles) had a half life of about 4 days. The half life of the 20,000 MW PEG-TNF-α (open triangles) was > 15 days.

This experiment was repeated using the treatment groups listed below, and the results presented in Fig. 2: native TNF-α (open circles); SS 5,000 MW PEG-TNF-α (closed triangles); SS 20,000 MW PEG-TNF-α (open triangles); NHS 12,000 MW PEG-TNF-α (closed squares). The serum half life for the different treatment groups was > 15 days for NHS 20,000 MW PEG-TNF-α and SS 20,000 MW PEG-TNF-α; approximately 4 days for SS 5,000 MW PEG-TNF-α; approximately 6 days for SS 12,000 MW PEG-TNF-α; approximately 8 days for NHS 12,000 MW PEG-TNF-α; and 30 min post-injection for native TNF-α. In summary, each PEG-TNF-α exhibited a much longer half life than native TNF-α; however, the NHS 20,000 MW PEG-TNF-α and the SS 20,000 MW PEG-TNF-α had significantly longer half lives (> 15 days) than the TNF-α modified with lower molecular weight PEG.

Antitumor Activity of PEG-TNF-α

The Bl6 melanoma model was used. C57 bl6 mice (female, 20-25 g) were injected with one million Bl6 cells, s.q. on flank. The tumors were allowed to grow for one week before beginning treatment. Each treatment group consisted of 5 mice.

The treatment groups included: phosphate buffer control (ph 7.5), native TNF-α (10 I.U. and 100 I.U.) in phosphate buffer (ph 7.5), and SS-PEG-TNF-α (10 I.U., 100 I.U. and 1000 I.U.) in phosphate buffer (ph 7.5). The mice were injected with 0.1 ml i.p. once a week on Day 7, Day 14, and Day 21. Survival of the animals was recorded.

After Week 2 (one week after first treatment), the tumors in the control animals were quite visible. None of the SS-PEG-TNF-α treated animals appeared to have tumors growing.

At Day 22, the results presented in Table 1 were obtained:

Table 1

Treatment Group	Results
Control	4 dead, 1 with large tumor
Native TNF-α	
10 I.U.	all animals dead
100 I.U.	4 dead, 1 with large tumor
5000 MW SS-PEG-TNF-α	
10 I.U.	2 dead, 3 with large tumors
100 I.U.	2 dead, 2 with tumors, 1 tumor-free
1000 I.U.	1 dead, 3 with small tumors, 1 tumor-free
20000 MW SS-PEG-TNF-α	
10 I.U.	0 dead, 1 with small tumor, 4 tumor-free
100 I.U.	all animals tumor-free
1000 I.U.	all animals tumor-free

After Day 180, the results presented in Table 2 were obtained.

Table 2

Treatment Group	Survival Time	Average
Control	18, 18, 20, 21, 24 days	20.2 days
Native TNF-α		
10 I.U.	17, 18, 19, 21, 21 days	20.2 days
100 I.U.	16, 18, 19, 19, 23 days	19.0 days
5000 MW SS-PEG-TNF-α		
10 I.U.	20, 22, 24, 26, 27 days	23.6 days
100 I.U.	21, 22, 24, 26, 27 days	35.0 days
1000 I.U.	21, 49, 53 days; 2 animals alive	
20,000 MW SS-PEG-TNF-α		
10 I.U.	38 days, 4 animals alive	
100 I.U.	all animals alive	
1000 I.U.	all animals alive	

These results indicate that modification of TNF with PEG not only reduces the lethality of the TNF but that the TNF modified with PEG having a molecular weight of approximately 20,000 exhibited surprisingly enhanced circulating half lives and surprisingly and significantly enhanced anti-tumor activity.

These results are surprising for a number of reasons. There was no way to predict that modifying TNF with high molecular weight PEG would increase the circulating half-life of the TNF. Indeed, the clearance rate of proteins in general cannot be predicted based on their molecular weight. That modification of TNF with high molecular weight PEG not only does not diminish, but actually enhances, the tumoricidal activity of the TNF is surprising in view of the added stearic hindrance expected to be created by the high molecular weight modifier. Still further, one would have predicted that the modified TNF, because of its enhanced circulating half life, would have been even more toxic than the native TNF, which was not the case.

Following is a description of the cytotoxicity assay utilized in this example.

A. Materials

L929 fibroblasts ATCC #CCL1 NCTC clone 929.

Dulbecco's Modified Essential Medium (DMEM)

Fetal Bovine Serum (GIBCO Laboratories, Grand Island, NY #16000-010)

HEPES Buffer 1M in Normal Saline (BioWhittaker, Inc. #17-737E)

Gentamicin Sulfate (BioWhittaker, Inc., #17-518Z)

Trypsin-EDTA 1X (GIBCO Laboratories, #25300-021)

Trypsin Blue Stain (GIBCO Laboratories, #15250-012)

Tissue culture flasks, 150cm^2, 75cm^2, 25cm^2 (Corning, Inc., Corning, NY, #25120, #25110, #25100)

96-Well cell culture plates, flat bottom (Corning Inc., #25861)

12-Channel pipetter (Titertek)

Sterile tips for pipetter (Intermountain Scientific Corp., Bountiful, UT, #P-3250-8)

Sterile reagent reservoirs (Costar Corp., Cambridge, MA, #4870)

Recombinant Human Tumor Necrosis Factor-α (TNF-α) (prepared in-house)

Albumin, bovine, 10% in IMEM (Boehringer Mannheim Corp., Indianapolis, IN, #652237)

Phosphate Suffered Saline (PBS)

Dulbecco's Phosphate Buffered Saline (D PBS) (GIBCO Laboratories, #310-4190)

Microtiter Plate Reader (Molecular Devices Corp., Menlo Park, CA, Emax)

B. Propagation of L929 Fibroblasts:

1. Maintain fibroblasts by passing twice weekly in DMEM/10% FBS as follows. Aspirate the medium from the flask, leaving the adherent cells. Wash the cell monolayer with 5 ml trypsin-EDTA and aspirate off the trypsin-EDTA. Incubate 1 to 2 minutes at 37°C. Add 15 ml DMEM/FBS to wash off the monolayer and stop the enzymatic activity of the trypsin.

2. Count the cells and evaluate viability by Trypan Blue exclusion. Inoculate 30 ml DMEM/FMS without antibiotics in a T75 flask with 1 X 10^6 cells. Incubate in a 37°C, 5% CO_2 humidified incubator.

3. For the assay, inoculate 60 ml DMEM/FBS in a T150 Flask with 2 X 10^6 cells. Incubate flask for 3 or 4 days until the cells approach confluency (80-90%)

C. TNF-α In Vitro Cytotoxicity Assay

Day One: Preparing the cells

1. Trypsinize cells and dilute with DMEM/FBS to a final concentration of 1.22 X 10^6 cells/ml. Add gentamicin sulfate to a final concentration of 50 μg/ml. A cell count of 2 X 10^6 cells are required for each plate, and 1.75-2 X 10^7 cells can be expected from a T150 flask.

2. Plate the L929 fibroblasts by adding 150 μl of the cell suspension to each well of a 96-well flat-bottom tissue culture plate. Incubate overnight in a 37°C, 5% CO_2 humidified incubator.

Day Two: Adding the samples

1. Using an inverted microscope, check the confluency of each well before proceeding. Each well must be equivalently confluent for the assay to be reproducible. The outside wells of the plate are not used for assay calculations due to the uneven growth of the cells in these wells. The cells must be 75 to 90% confluent for best sensitivity. As the passage number of the cells increases, the hours per doubling of the cells decreases. The cell number/well can be adjusted accordingly to obtain the correct confluency.

2. Prepare TNF-α standard working solution. Keep at 4°C until use.

3. Add 150 μl of the TNF working solution to the first well of columns 2 and 3. Add 150 μl of sample to the first well of Columns 4 through 10. Add DMEM/FBS/gentamicin to Columns 1, 11, and 12. Make 2-fold serial dilutions by mixing the contents of the first wells and transferring 150 μl to the second wells of the columns, using a 12-channel pipetter. Finally, mix the contents of the 8th wells (row H) and discard 150 μl form each well. At this point, all wills should contain 150 μl.

4. Incubate the plates 20 hours in a 37°C, 5% CO_2 humidified incubator.

Day three:

Viability of the cells was determined by adding 20 μl of 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (25 mg/ml in phosphate buffered saline pH 7.4) to each well of the culture plate and incubating the cultures at 37°C for four hours. After that time, the culture supernatents were discarded and 150 μl of DMSO was added to each well. The absorbance of each well at 570 nm was

determined using a micro titer plate reader. Wells that exhibit an A_{540} closest to 50% of the arithmetic mean of the control are considered to represent 50% lysis (1 unit) of the L929 cells.

D. SOLUTIONS

TNF-α Standard (10 μg)

Thaw frozen TNF-α standard on ice. Aliquot into screw cap cryotubes at 2 μg/tube and store at -80°C.

TNF-α Standard Stock Solution (256 ng/ml)

To one tube (2μg) of TNF-α standard, add 7.81 ml 1% albumin in D-PBS/gentamicin. Aliquot into 1 ml tubes and keep at 4°C. Solution is typically used for six months.

TNF-α Working solution (0.8 ng/ml)

For two plates, add 3.12 μl stock solution to 997 μl DMEM/FBS/gentamicin. (The standard solution will be diluted 1:2 in the first well of the multiwell plate for a final concentration of 0.4 ng/ml). Keep at 4°C until use.

Phosphate Buffered Saline 1L (PBS, 1x)

Dissolve 0.2 g Kcl, 0.2 g KH_2PO_4, 8 g NaCl, and 1.14 g Na_2HPO_4 in 900 ml water. Adjust pH to 7.4 Q.S. to 1 L. Autoclave.

Example 2

Additional samples of human recombinant TNF modified with PEG were prepared (according to general methods described in Harras, *Supra.*) and tested for their serum half life and for retention of specific activity. In each case, the TNF was modified with about five to nine molecules of PEG. The data (Table 3) show, surprisingly, that the TNF modified with PEG having an approximate molecular weight average of about 10000 to 40000, preferably about 20000-30000, exhibit a significantly enhanced serum half life while retaining high specific activity.

The serum half-life of the various formulations of TNF were determined using ELISA assays obtained from Genzyme (Cambridge, MA), as suggested by the manufacturer. Serum samples were collected from retro orbital plexus using heparinized 50μl capillary tubes. A pretreatment blood sample was collected just prior to i.v. injection with TNF or PEG-TNF formulations. Additional blood samples were collected at 30

minutes, 24 hours as well as 3, 7, 12 and 15 days post-treatment. The samples were centrifuged and the resulting supernatant was stored frozen at -20°C until being assayed.

Table 3

Treatment	Serum Half Life (days)	% Specific Activity Retained
SS-PEG 5000 TNF-α (2 tests)	4, 4	55, 58
SS-PEG 12000 TNF-α (2 tests)	8, 8	52, 53
SS-PEG 20000 TNF-α	16	56
SS-PEG 30000 TNF-α	17	54
SS-PEG 40000 TNF-α	17	55
SP-PEG 5000 TNF-α	5	51
SP-PEG 20000 TNF-α	8	53
Branched chain succinimide-PEG 10000 TNF-α	7	49
Branched chain succinimide-PEG 20000 TNF-α	16	52
Branched chain succinimide-PEG 40000 TNF-α	18	54

Example 3

Additional samples of PEG modified, human recombinant TNF were prepared and tested for serum half life and specific activity retention. The data presented in Table 4 show that half-life and specific activity can vary dependent upon the linker utilized.

Table 4

Treatment (#PEG's/TNF)	Serum Half Life (days)	% Specific Activity Retained
SCM-PEG 5000 TNF-α (5-9 PEG's)	5	54
Succinimidyl ester of amino acid-PEG 5000 TNF-α (6-8 PEG's)	4	55
Epoxide PEG 8000 TNF-α (primary amine attachment site) (9-12 PEG's)	6	38
Epoxide PEG 8000 TNF-α (hydroxyl attachment site) (10-20 PEG's)	5	0
Glycidyl ether PEG 5000 TNF-α (15 PEG's)	12	0
Nitrophenyl PEG 5000 TNF-α (6-9 PEG's)	5	21
Trichlorophenyl carbonate PEG 5000 TNF-α (12-15 PEG's)	5	11
PEG tresylate 5000 TNF-α (10-12 PEG's)	2	8
PEG aldehyde 5000 TNF-α (1 PEG)	<1	100
PEG aldehyde 20000 TNF-α (1 PEG)	2	100
PEG isocyanate 5000 TNF-α (5-12 PEG's)	9	19
PEG vinylsulfone 5000 TNF-α (4 PEG's)	3	12
PEG maleimide 5000 TNF-α (3 PEG's)	3	43

Example 4

Additional tests were done to evaluate the effect of PEG-modified, recombinant human TNF on mice injected with different varieties of tumor cells. The TNF utilized in these tests was modified with SS-PEG 20000 according to a method as described Harras, *Supra*. Mice were injected with 1 x 10^6 tumor cells and, two weeks later, were injected i.p. with the PEG-TNF once a week, for three weeks. Cure was defined as the percent of animals surviving five times longer than untreated animals. Results are presented in Table 5 and indicate that the modified TNF of this invention is effective in treating melanoma tumors, kidney tumors, colon tumors, and breast tumors.

Table 5

Tumor Type	Cell Line	Dose of PEG-TNF I.U.	% Cure
Melanoma	B16	10	75
		30	100
		100	100
Kidney	G401	10	80
		30	80
Colon	HT29	10	40
		30	60
		100	80
Breast	MCF7	10	0
		30	0
		100	20
Brain	SW1088	100	0
Leukemia	L1210	100	0
Hepatoma	Hep3B	100	0

Example 5

Tests were done to evaluate TNF from a variety of sources, modified with PEG. Results are presented in Table 6.

Table 6

TNF PROTEIN	SPECIFIC ACTIVITY	LD50	Hypotension ED50	Antitumor ED50
Recom. murine TNF produced in Pichea	$2.1x10^7$ IU/mg	20μg	1μg	> 1000 IU
" - modified with SS-PEG MW 20000	$1.0x10^7$ IU/mg	100μg	2μg	20 IU
Recom. murine TNF produced in E. coli	$2.0x10^7$ IU/mg	70μg	1μg	> 3000 IU
" - modified with SS-PEG MW 20000	$1.0x10^7$ IU/mg	300μg	4μg	20 IU
Human TNF mutated by deletion of amino acids 212-220	$2.1x10^7$ IU/mg	60μg	1μg	> 2000 IU
" - modified with SS-PEG MW 20000	$0.9x10^7$ IU/mg	100μg	5μg	10 IU
Human TNF mutated by changing 248, 592, 508 lysine to alanine	$1.5x10^7$ IU/mg	300μg	100μg	> 1000 IU
" - modified with SS-PEG MW 20000	$0.5x10^7$ IU/mg	> 1000μg	> 100μg	5 IU

WHAT IS CLAIMED IS:

1. Modified TNF, comprising TNF covalently bound to between about five and twelve PEG molecules having an approximate weight average molecular weight in the range of about 10,000 to about 40,000.

2. The modified TNF of Claim 1 wherein said PEG has an approximate weight average molecular weight in the range of about 20,000 to about 30,000.

3. The modified TNF of Claim 1 wherein said PEG is covalently bonded to said TNF through a biocompatible linker.

4. The modified TNF of Claim 1 wherein said linker is N-hydroxysuccinimidyl succinate.

5. The modified TNF of Claim 1 wherein said linker is N-succinimidyl propionate.

6. The modified TNF of Claim 1 wherein said TNF is covalently bound to about five to nine PEG molecules.

7. The modified TNF of Claim 1 wherein said TNF is covalently bound to said PEG molecules through primary amines on the TNF.

8. The modified TNF of Claim 1 wherein said TNF is TNF-α.

9. The modified TNF of Claim 1 wherein said TNF is isolated human TNF.

10. The modified TNF of Claim 1 wherein said TNF is recombinant human TNF.

11. A method of enhancing the circulating half life of TNF comprising modifying said TNF by covalently bonding to it between about five and twelve PEG molecules having an approximate weight average molecular weight in the range of about 10,000 to about 40,000.

12. A method of enhancing the tumoricidal activity of TNF comprising modifying said TNF by covalently bonding to it between about five and twelve PEG molecules having an approximate

13. A method of treating a patient suffering from a tumor comprising administering to said patient a therapeutically effective amount of the modified TNF of Claim 1.

14. The method of Claim 13 wherein said tumor is a melanoma.

15. The method of Claim 13 wherein said tumor is a colon cancer.

16. The method of Claim 13 wherein said tumor is a kidney cancer.

17. The method of Claim 13 wherein said tumor is a breast cancer.

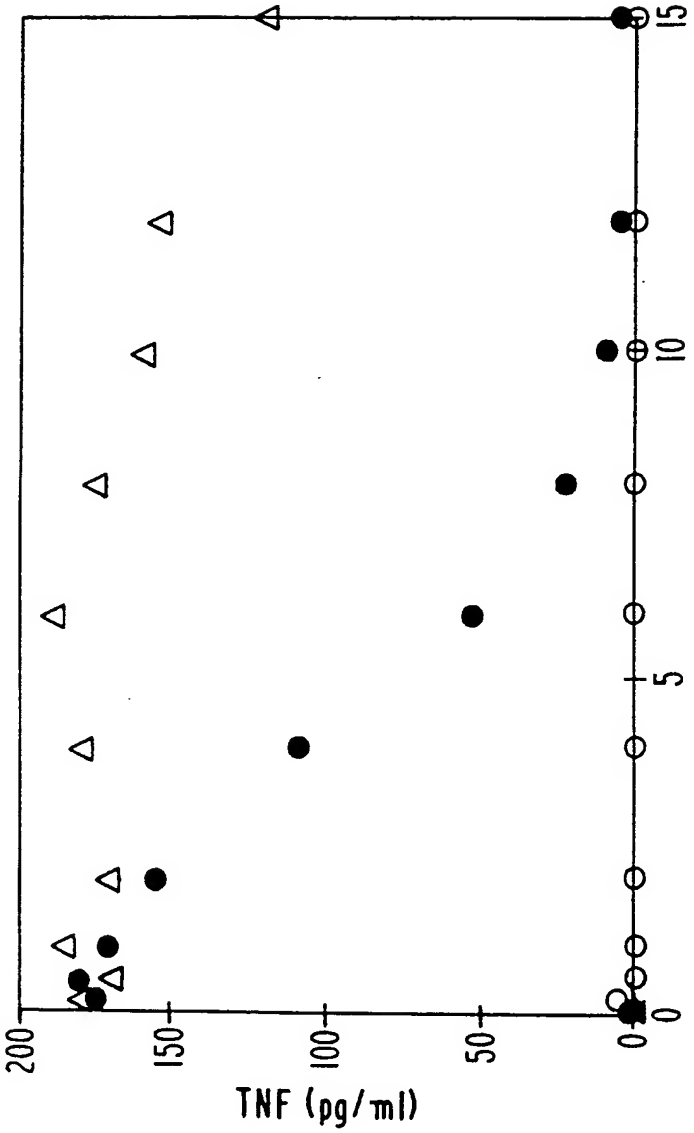

Fig. 1

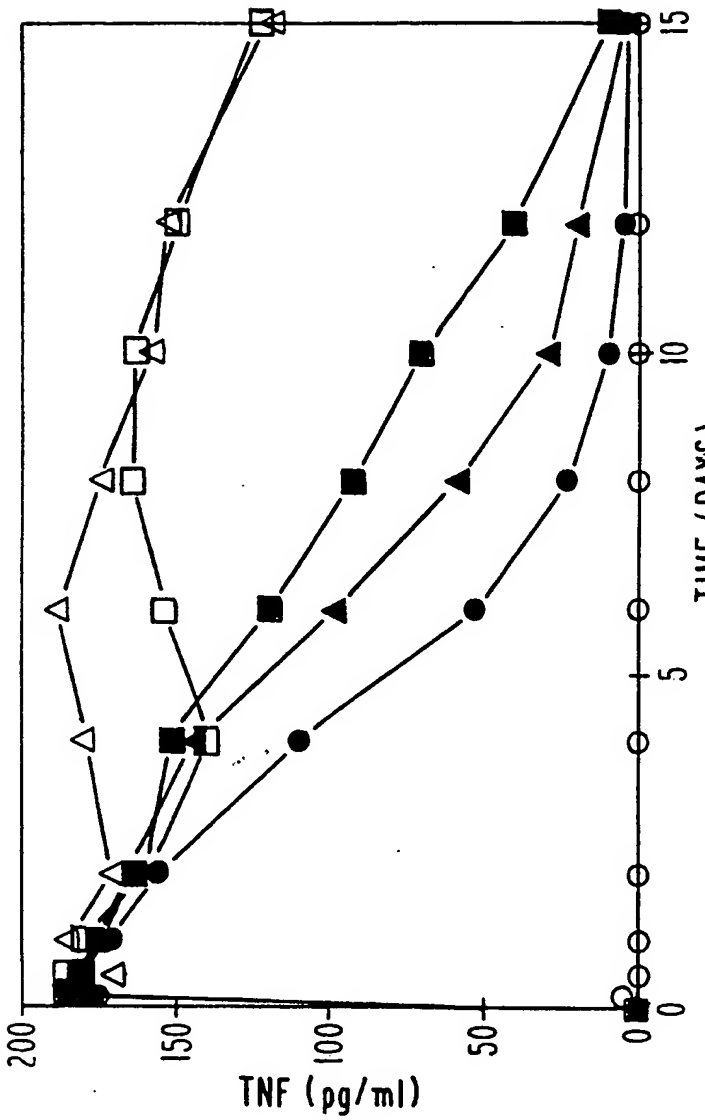

Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/00683

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 38/19; C07K 14/525
US CL :424/85.1; 514/12; 530/350, 399, 402
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/85.1; 514/12; 530/350, 399, 402

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
APS, CAPLUS
search terms: succinimidyl succinate or propionate, polyethyleneglycol, tnf, tumor necrosis factor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	TSUTSUMI et al. Chemical modification of natural human tumor necrosis factor alpha with polyethylene glycol increases its anti-tumor potency. Jap. J. Cancer Res. January 1994, Vol 85, No. 1, pages 9-12, especially Abstract, full paragraphs 1-3 on page 9, full paragraph 2 on page 10, full paragraph 2 on page 11.	1, 3, 4, 6-9, 11-13 -- 2, 5, 10, 14-17
Y	PENNICA et al. Human tumor necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature. 27 December 1984, Vol. 312, pages 724-729, especially page 727 full paragrph 5 through page 728 full paragraph 1.	10
Y	US 5,264,209 A (MIKAYAMA et al.) 23 November 1993, paragraph bridging columns 2-3.	1, 5

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
26 MARCH 1998

Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/00683

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US 5,695,760 A (FAANES et al.) 09 December 1997, col. 12, full paragraph 2.	1-11
Y	US 4,640,835 A (SHIMIZU et al.) 03 February 1987, Abstract.	2